Interleukin-12- and interferon-γ-mediated natural killer cell activation by *Agaricus blazei* Murill

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Summary

Dried fruiting bodies of *Agaricus blazei* Murill (*A. blazei*) and its extracts have generally used as complementary and alternative medicines (CAMs). Here, we report that the oral administration of *A. blazei* augmented cytotoxicity of natural killer (NK) cells in wild-type (WT) C57BL/6, C3H/HeJ, and BALB/c mice. Augmented cytotoxicity was demonstrated by purified NK cells from treated wild-type (WT) and RAG-2-deficient mice, but not from interferon-γ (IFN-γ) deficient mice. NK cell activation and IFN-γ production was also observed *in vitro* when dendritic cell (DC)-rich splenocytes of WT mice were coincubation with an extract of *A. blazei*. Both parameters were largely inhibited by neutralizing anti-interleukin-12 (IL-12) monoclonal antibody (mAb) and completely inhibited when anti-IL-12 mAb and anti-IL-18 mAb were used in combination. An aqueous extract of the hemicellulase-digested compound of *A. blazei* particle; (ABPC) induced IFN-γ production more effectively, and this was completely inhibited by anti-IL-12 mAb alone. NK cell cytotoxicty was augmented with the same extracts, again in an IL-12 and IFN-γ-dependent manner. These results clearly demonstrated that *A. blazei* and ABPC augmented NK cell activation through IL-12-mediated IFN-γ production.

Keywords: *Agaricus blazei*; NK cells; IFN-γ; IL-12; cytotoxicity

Introduction

Agaricus blazei Murill (*A. blazei*), a Brazilian native edible mushroom, has been used as a health food or a non-prescription remedy in traditional medicine for preventing cancer, diabetes, hyperlipidaemia, arteriosclerosis and chronic hepatitis in Brazil.[1] *A. blazei* and the hemicellulase-digested component of *A. blazei* particle compound (ABPC) have recently been used as one of the common complementary and alternative medicines (CAMs).[1–3] Oral intake of extracts of *A. blazei* has been reported to improve the living quality of cancer patients particularly undergoing chemotherapy.[2,4] The antitumour effect of *A. blazei* has been demonstrated in several transplantable mouse tumour models.[1,5–11] In addition, some reports suggested that augmentation of cellular immune responses is a critical mechanism for their antitumour effect.[5] Particularly, natural killer (NK) cells, which play an important role in innate immunity against infection and tumour development[12–15] have been reported to be activated by *A. blazei in vivo* and *in vitro*.[5,10,16–18] The administration of the extract of *A. blazei* has been reported to restore NK cell activity in tumour bearing mice or cancer patients treated with chemotherapy.[4,18] However, the precise mechanisms of NK cell activation by *A. blazei* have still not been clearly revealed, especially in oral administration models using experimental mice. Here we examined the effect of *A. blazei* and ABPC on NK cell cytotoxicty using gene-targeted mice, and demonstrated that *A. blazei* and ABPC induced interleukin-12 (IL-12)-mediated interferon-γ (IFN-γ) production and augmented NK cell cytotoxicity on a per cell basis.

Abbreviations: *A. blazei*, *Agaricus blazei* Murill; CAM, complementary and alternative medicine; NK, natural killer; WT, wild type; IFN, interferon; DC, dendritic cell; IL, interleukin; mAb, monoclonal antibody; ABPC, *Agaricus blazei* particle compound; MNC, mononuclear cell; ELISA, enzyme-linked immunosorbent assay; TLR, Toll-like receptor.

Materials and methods

Mice

Wild-type (WT) male C57BL/6 (B6), C3H/HeJ, and BALB/c mice, 6 weeks of age, were purchased from Charles River Japan Inc. (Yokohama, Japan). IFN-γ-deficient (IFN-$\gamma^{-/-}$) and Rag-2-deficient (RAG-$2^{-/-}$) B6 mice were derived as described previously.[19] All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University.

Reagents

Powdered dried fruiting bodies of *A. blazei* Murrill (H1) (*A. blazei*) and the hemicellulase-digested component of *A. blazei* particle (ABPC)[20] were kindly provided from Japan Applied Microbiology Research Institute Ltd (Yamanashi, Japan). These are orally administered into mice as a suspension in the distilled water (500 μl). Extracts were prepared with distilled water at 37° for 1 hr at 200 mg/ml, and supernatants were passed through a 0·22 μm filter (Millipore Co., Bedfold, MA) after centrifugation at 2000 ***g*** for 15 min. The approximate content of β-glucan as estimated by a colorimetric analysis using *G*-test (Medical & Biological Laboratories, Nagoya, Japan) was 380 ng/ml in *A. blazei* extract and 820 ng/ml in the ABPC extract. Endotoxin was not detected in either extract by an analysis using an endotoxin-specific chromogenic *Limulus* test (Wako Biochemicals, Osaka, Japan). *Eschericha coli*-derived lipopolysaccharide (LPS) and polymixin B were purchased from Sigma Chemical (St Louis, MO). Neutralizing anti-mouse IL-12 monoclonal antibody (mAb) (C17.8) was purchased from eBioscience (San Diego, CA), and neutralizing anti-mouse IL-18 mAb (93-10C) was purchased from Medical & Biological Laboratories. Anti-mouse IL-12 mAb (C17.8) was also prepared and purified from ascites using protein G column in our laboratory for *in vivo* usage as previously described.

Cytotoxicity assay and purification of NK cells

Liver mononuclear cells (MNCs) were prepared as previously described.[21] In some experiments, freshly isolated liver MNCs were incubated with anti-DX5 microbeads (Miltenyi Biotec, Bergisch Glabach, Germany), and DX5^{+} cells were enriched or eliminated by auto-magnetic-activated cell sorting (Miltenyi Biotec) according to the manufacturer's instructions. Flow cytometric analysis demonstrated more than 90% pure NK cell populations and less than 2% of NK cells in the NK cell-depleted population. Cytotoxic activity of MNCs and purified liver NK cells was assessed against the NK cell-sensitive target, YAC-1 cells, by a standard ^{51}Cr release assay.[21]

Flow cytometric analysis

After preincubation with anti-mouse CD16/32 mAb (2.4G2) to avoid non-specific binding of mAbs to Fcγ receptors, cell surface molecules were stained with fluoroscein isothiocyanate-conjugated anti-mouse CD3 mAb (145-2C11) and phycoerythin-conjugated anti-NK1.1 mAb (PK136) and analysed using a FACS Caliber (BD Bioscience, San Jose, CA).[21] All reagents were purchased from eBioscience.

In vitro *culture of splenic MNCs with an extract of* A. blazei

Dendritic cell (DC)-rich splenic MNCs or splenic DCs were prepared according to reported procedures.[22,23] Briefly, spleen cells were digested with collagenase (400 U/ml, Wako Biochemicals) in the presence of 5 mM EDTA in Ca^{2+}-free media, and red blood cells were lysed. In some experiments, DCs were purified by cell density from DC-rich splenocytes. Peritoneal macrophages were isolated using the previously reported method.[22] Cells (1×10^5 cells/200 μl) were cultured with titrated extract of *A. blazei* or ABPC in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 25 mM $NaHCO_3$ in humidified 5% CO_2 at 37° on 96-well flat-bottom culture plate (Costar, Cambridge, MA). Cell-free supernatants were harvested 72 hr later. In some experiments cells were cultured in the presence of anti-IL-12 mAb and/or anti-IL-18 mAb (10 μg/ml). In some experiments, extracts and LPS were incubate with 10 μg of polymixin B for 1 hr before culture.

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ or IL-12 p40 levels in the culture supernatants were evaluated by using a highly sensitive mouse IFN-γ specific ELISA kit (Ready-SET-Go!; eBioscience) or IL-12 p40-specific ELISA kit (OptEIA; BD PharMingen) according to the manufacturer's instruction.

Statistical analysis

Data were analysed by a two-tailed Student *t*-test. *P*-values less than 0·05 were considered significant.

Results

Augmentation of NK cell cytotoxic activity by oral administration of *A. blazei*

To investigate the effect of *A. blazei* administration on the cytotoxic activity of mouse MNCs in a model possibly analogous to the usage of *A. blazei* in humans, we orally administered a suspension of powdered dried fruiting

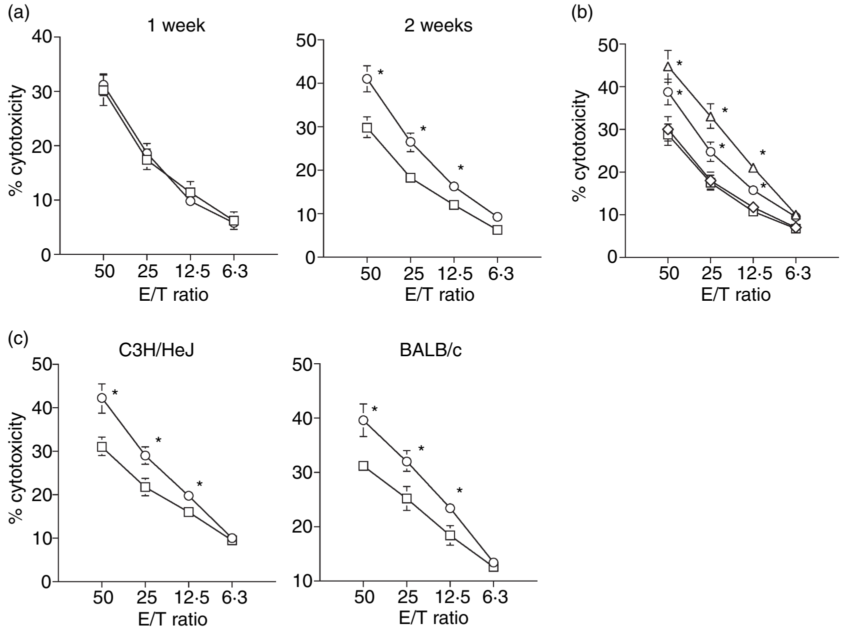

Figure 1. Activation of NK cell cytotoxicity by oral administration with *A. blazei*. (a) B6 mice were administered daily with *A. blazei* suspension (32 mg/500 μl/head) (open circle) or water (500 μl/head) (open square) for 1 or 2 weeks. Then, liver MNCs were prepared and cytotoxic activity was analysed using NK cell-sensitive target cells, YAC-1, at indicated E/T ratios. (b) B6 mice were administered daily with 500 μl of suspension containing 0 mg (open square), 16 mg (open diamond), 32 mg (open circle), or 64 mg (open triangle) of *A. blazei* for 2 weeks. Then, liver MNCs were prepared and cytotoxic activity was analysed using NK cell-sensitive target cells, YAC-1. (c) C3H/HeJ and BALB/c mice were administered daily with *A. blazei* suspension (32 mg/500 μl/head) (open circle) or water (500 μl/head) (open square) for 2 weeks. Then, liver MNCs were prepared and cytotoxic activity was analysed using NK cell-sensitive target cells, YAC-1, at indicated E/T ratios. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in two or three independent experiments. *$P < 0.05$.

bodies of *A. blazei* into mice. Daily administration of 32 mg of *A. blazei* into WT B6 mice for 2 weeks, but not 1 week, augmented cytotoxicity of liver MNCs against the NK cell-sensitive target, YAC-1 (Fig. 1a). Daily administration of 32 mg or 64 mg of *A.blazei* significantly augmented the cytotoxic activity of liver MNCs, although daily intake of 16 mg of *A. blazei* did not augment cytotoxic activity (Fig. 1b). To exclude the contribution of LPS, which possibly contaminates powdered dried fruiting bodies of *A. blazei*, we also administered the *A. blazei* suspension into Toll-like receptor (TLR)-4-defective C3H/HeJ mice.[24] Oral administration of *A. blazei* augmented the NK cell cytotoxicity of liver MNCs in C3H/HeJ mice as well as in BALB/c mice (Fig. 1c). The NK cell activity of spleen MNCs was also increased by oral administration *of A. blazei* in all strains of mice tested (data not shown). However augmentation of NK cell cytotoxicity in spleen MNCs was weaker compared with that of liver MNCs, which might be caused by a reduced proportion of NK cells in spleen compared with liver. In spite of the significant augmentation of NK cell cytotoxicity by the oral administration of *A. blazei*, neither MNC numbers nor populations of NK cells significantly increases in liver and spleen (Figs 2a, b and data not shown). No signs of hepatotoxicity with significantly elevated serum transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], or systemic toxicity as estimated by body weight, gross appearance, or behaviour was observed in all mice orally administered with a suspension of powdered dried fruiting bodies of *A. blazei* (data not shown). These results showed that NK cell cytotoxicity, but not NK cell number, was increased independent of TLR-4 when *A. blazei* were orally administered daily for 2 weeks.

Requirement of IFN-γ but not T cells or NKT cells, in *A. blazei*-induced NK cell activation *in vivo*

To investigate the contribution of T cells, NKT cells, and IFN-γ to *A. blazei*-induced cytotoxicity *in vivo*, we next orally administered *A. blazei* into RAG-$2^{-/-}$ or IFN-$\gamma^{-/-}$ mice. NK cells purified from liver MNCs, isolated from WT and RAG-$2^{-/-}$ mice 2 weeks after daily administration of *A. blazei*, displayed augmented cytotoxicity (Fig. 3), suggesting that NK cells were the cytotoxic effector cells. Alternatively, the cytotoxicity of NK cells purified from IFN-$\gamma^{-/-}$ mice was not augmented (Fig. 3), indicating a critical role for IFN-γ in the augmentation of NK cell activity by *A. blazei in vivo*. Similar results were obtained in the experiments using whole liver or spleen MNCs or when ABPC was orally administered into these mice (data not shown). These results clearly indicated that oral administration with *A. blazei* augmented NK cell cytotoxi-

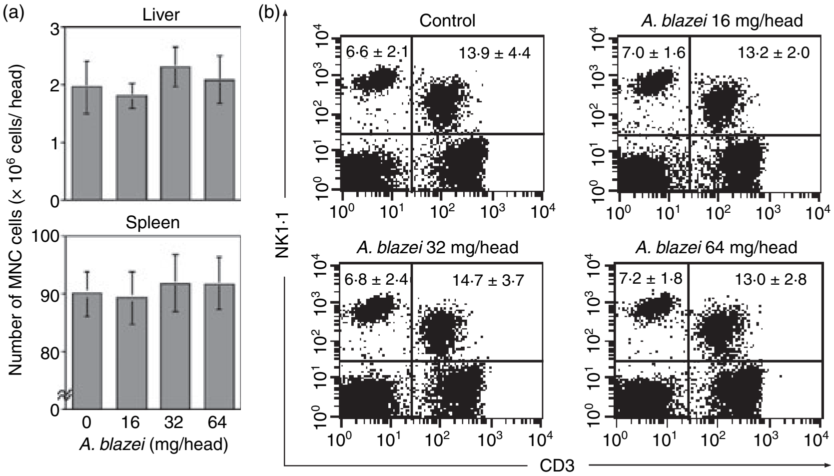

Figure 2. MNC numbers and NK cell percentage after *A. blazei* oral administration for 2 weeks. Two weeks after daily oral administration of *A. blazei* suspension (32 mg/500 μl/head) into WT B6 mice, spleen and liver MNCs number (a) and populations of liver MNC were analyzed with flow cytometry (b). Data are shown as mean ± SD of three to five mice in each group. Similar results were obtained in two independent experiments.

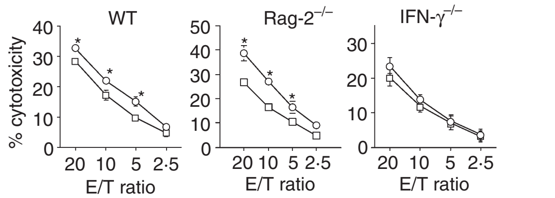

Figure 3. IFN-γ dependent NK cell activation by oral administration of *A. blazei*. WT, RAG-2$^{-/-}$, and IFN-γ$^{-/-}$ B6 mice were orally administered daily with *A. blazei* (32 mg/500 μl/head) (open circle) or water (500 μl/head) (open square) for 2 weeks. Then, NK cells were purified from liver MNCs and cytotoxic activity was analysed using NK cell-sensitive target cells, YAC-1, at indicated E/T ratios. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. *$P < 0{\cdot}05$.

city on a per cell basis and this was dependent on IFN-γ but not T or NKT cells.

IFN-γ production by DC-rich spleen MNC stimulated *in vitro* with an extract of A. blazei or ABPC

To further examine the mechanisms of NK cell activation and IFN-γ production induced by *A. blazei*, we used *in vitro* culture experiments employing the extract from *A. blazei* or ABPC. Aqueous extract of *A. blazei* induced IFN-γ production by DC-rich spleen MNCs after 72 hr incubation, and the peak of IFN-γ production was observed when cells were cocultured with a 1/1000 diluted extract (Fig. 4a). ABPC extract induced similar amount of IFN-γ production when cells were cocultured with a 1/2000 diluted (Fig. 4a). Both extracts induced IFN-γ production from DC-rich spleen MNCs isolated from B6 mice and TLR-4-defective C3H/HeJ mice[24] (Fig. 4a). Even when DC-rich spleen MNC were stimulated with the extract of *A. blazei* or ABPC in the presence of LPS-inactivating polymixin B[25] a similar amount of IFN-γ was detected in culture supernatants (Fig. 4b). Polymixin B almost completely diminished IFN-γ production (less than 10 pg/ml) induced by *in vitro* stimulation with 5 μg/ml of LPS (Fig. 4c). IFN-γ was detected in the supernatants of DC-rich spleen MNCs prepared from RAG-2$^{-/-}$ mice when stimulated with either extract *in vitro*; however, IFN-γ was not detected when DC-rich spleen MNCs were stimulated after NK cell depletion (Fig. 4d). Thus, these results clearly suggested that the extract of *A. blazei* or ABPC induced IFN-γ production from NK cells independently of LPS and TLR-4 *in vitro*.

Dominant role of IL-12 in IFN-γ production by DC-rich spleen MNC stimulated with an extract of *A. blazei* or ABPC

We examined IL-12 production from DCs and macrophages, because it has been reported that IL-12 was produced by CD14^{+} human monocytes/macrophages stimulated *in vitro* with the extract of ABPC.[20] IL-12 was detected in the culture supernatants of splenic DCs stimulated with 0·1% of *A. blazei* extract or 0·05% of ABPC extract (Fig. 5a). In addition, the amount of IL-12 produced by peritoneal macrophages stimulated with either extract was extract dose dependent (Fig. 5b). To confirm the contribution of IL-12 to IFN-γ production, we examined IFN-γ production from DC-rich spleen cell cultured with the extract in the presence of a neutralizing anti-IL-12 mAb. As shown in Fig. 6(a), neutralization of IL-12 completely inhibited IFN-γ production when cells were stimulated with the extract of ABPC. Alternatively, a small amount of IFN-γ was still produced by DC-rich spleen cells stimulated with 0·1% of *A. blazei* extract even in the presence of anti-IL-12 mAb (Fig. 6a); however, this IFN-γ production was completely inhibited by coincubation with anti-IL-12 mAb and anti-IL-18 mAb (Fig. 6b). These results suggested that IL-12 plays the predominant role in IFN-γ induction by both extracts; however, IL-18 also contributes to IFN-γ induction by the *A. blazei* extract.

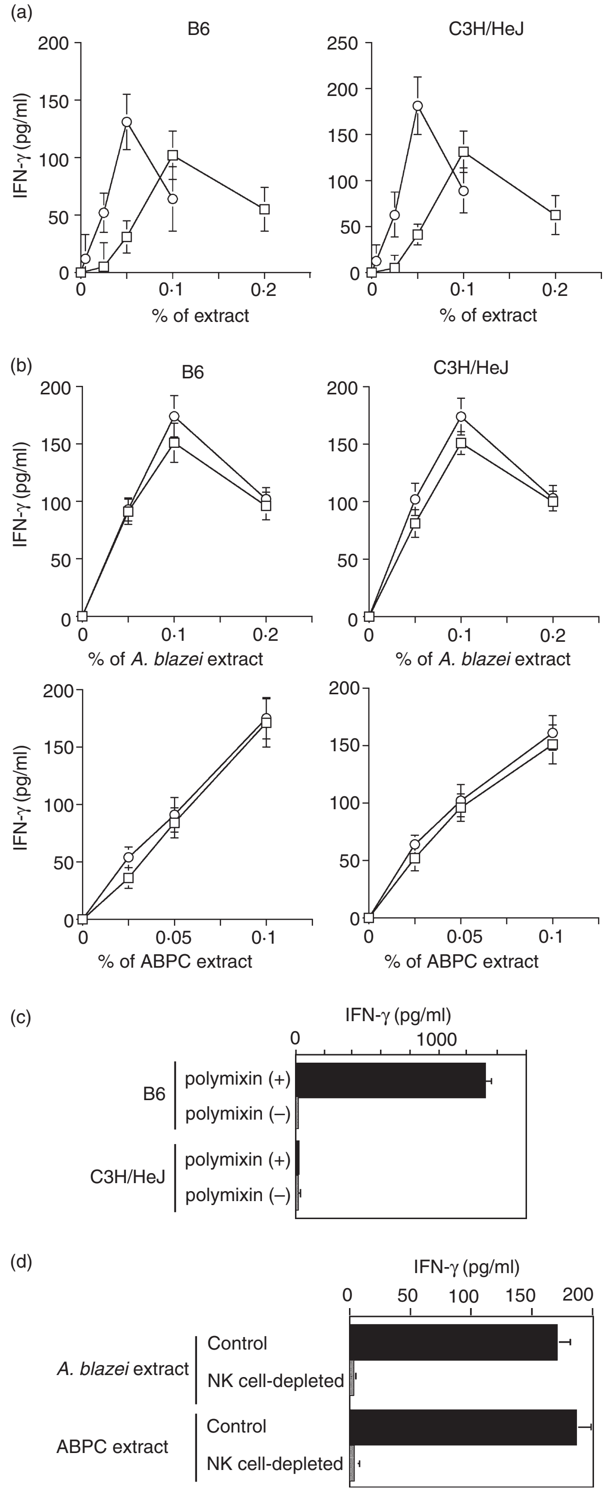

Figure 4. IFN-γ induction *in vitro* by an extract of *A. blazei* or ABPC. (a) DC-rich spleen MNCs isolated from B6 or C3H/HeJ mice were coincubated with an extract of *A. blazei* (open square) or ABPC (open circle) at the indicated concentrations for 72 hr. Cell free culture supernatants were then collected and IFN-γ was measured by ELISA. (b) LPS independent IFN-γ induction. DC-rich spleen MNCs isolated from B6 or C3H/HeJ mice were coincubated with an extract of *A. blazei* or ABPC in the presence (10 μg/ml) (open circle) or absence (open square) of polymixin B for 72 hr. Cell free culture supernatants were then collected and IFN-γ was measured by ELISA. (c) Sufficient inhibition by polymixin B on IFN-γ production induced by LPS. DC-rich spleen MNCs isolated from B6 or C3H/HeJ mice were coincubated with LPS (5 μg/ml) in the presence (10 μg/ml) or absence of polymixin B for 72 hr. Cell free culture supernatants were then collected and IFN-γ was measured by ELISA. (d) Major role of NK cells in IFN-γ production. NK cell-depleted or intact DC-rich spleen MNCs isolated from RAG-$2^{-/-}$ B6 mice were coincubated with an extract of *A. blazei* (0·1%) or ABPC (0·05%) for 72 hr. Cell free culture supernatants were then collected and IFN-γ was measured by ELISA. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in two or three independent experiments.

IL-12- and IFN-γ-dependent NK cell activation in DC-rich spleen MNC stimulated with an extract of *A. blazei* or ABPC *in vitro*

We then examined NK cell cytotoxic activity 72 hr after coincubation of DC-rich spleen MNC with the extracts of *A. blazei* or ABPC. When DC-rich splenocytes derived from WT mice were coincubated with 0·1% of *A. blazei* extract or 0·05% of the ABPC extract, NK cell cytotoxicity was significantly augmented (Fig. 7a), although NK cell proportions were not increased (data not shown). Cytotoxicity was not augmented when DC-rich spleen MNC isolated from IFN-γ-deficient mice were stimulated with extract of *A. blazei* or ABPC (Fig. 7b), although IL-12 was detected in the supernatants (data not shown). Moreover, cytotoxic activity was not augmented when NK cells were depleted or when DC-rich spleen MNC were coincubated with the extracts in the presence of anti-IL-12 mAb (Fig. 7c). These results clearly indicated that the extracts of *A. blazei* or ABPC induced IL-12, and this was critical for IFN-γ-dependent augmentation of NK cell cytotoxicity *in vitro*.

IL-12-dependent NK cell cytotoxic activation by oral administration of *A. blazei* or ABPC extract

We finally analysed NK cell cytotoxicity in B6 mice administered orally with the extract of *A. blazei* or ABPC for 2 weeks. Serum IL-12 was not detected during entire period of the administration of either extract (data not shown). However NK cell cytotoxicity of liver MNCs was augmented in the mice treated with either extract, and the augmentation of NK cell cytotoxicity was almost completely diminished by *in vivo* anti-IL-12 mAb treatment (Fig. 8a). Moreover, NK cell activation was not observed in IFN-$\gamma^{-/-}$ mice treated with extract of *A. blazei* or ABPC for 2 weeks (Fig. 8b). These data clearly showed that NK cell activation by oral administration of the extracts of *A. blazei* or ABPC is dependent on IL-12 and

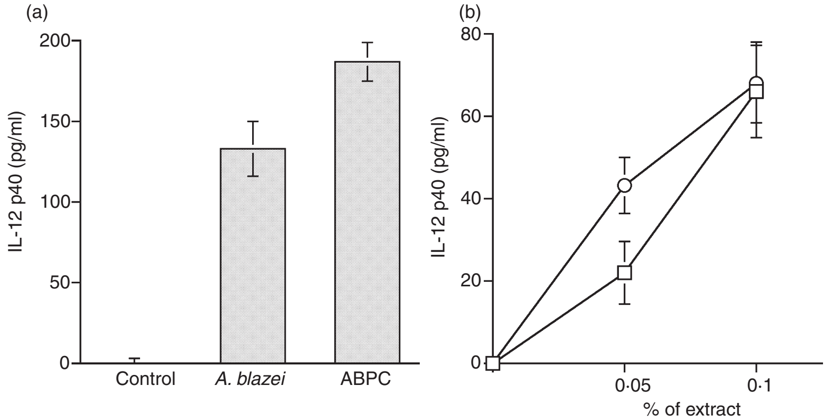

Figure 5. IL-12 production by DC or purified peritoneal macrophages stimulated with an extract of *A. blazei* or ABPC. (a) DCs were coincubated with an extract of *A. blazei* (0·1%) or ABPC (0·05%) for 72 hr. Cell free culture supernatants were then collected and IL-12 p40 was measured by ELISA. (b) Purified peritoneal macrophages were coincubated with en extract of *A. blazei* (open square) or ABPC (open circle) at the indicated concentrations for 72 hr. Cell free culture supernatants were then collected and IL-12 p40 was measured by ELISA. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

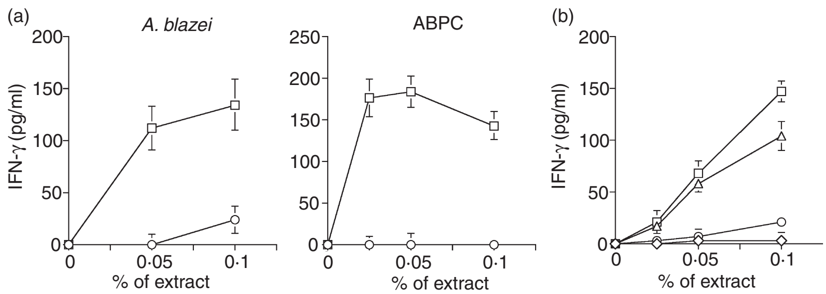

Figure 6. IL-12- and IL-18-dependent IFN-γ induction by *A. blazei*. (a) DC-rich spleen MNCs isolated from B6 mice were coincubated with an extract of *A. blazei* or ABPC at the indicated concentration in the presence (open circle) or absence (open square) of anti-IL-12 mAb for 72 hr. Cell free culture supernatants were then collected and IFN-γ was measured by ELISA. (b) DC-rich spleen MNCs isolated from B6 mice were coincubated with an extract of *A. blazei* at the indicated concentration in the presence of anti-IL-12 mAb (open circle), anti-IL-18 mAb (open triangle), anti-IL-12 mAb and anti-IL-18 mAb (open diamond), or control immunoglobulins (open square) for 72 hr. Cell free culture supernatants were then collected and IFN-γ was measured by ELISA. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

IFN-γ, consistent with the data obtained in *in vitro* experiments.

Discussion

In this study, we explored the activation of NK cell cytotoxicity by *A. blazei* or ABPC, utilizing RAG-$2^{-/-}$ mice, IFN-$\gamma^{-/-}$ mice and neutralizing mAbs. In RAG-$2^{-/-}$ mice but not in IFN-$\gamma^{-/-}$ mice, oral consumption of *A. blazei* augmented NK cell cytotoxicity as potently as in WT mice. Consistently, coincubation of DC-rich spleen MNC with an extract of either *A. blazei* or ABPC augmented NK cell cytotoxicity dependent upon IFN-γ. IFN-γ induction was mostly mediated by IL-12, although IL-18 partly contributed to IFN-γ production induced by the extract of *A. blazei*. Moreover, oral administration of either *A. blazei* or ABPC extract induced NK cell activation; however, it was not observed in anti-IL-12 mAb-treated WT mice or IFN-$\gamma^{-/-}$ mice. This is the first report clearly showing that *A. blazei* or ABPC augment NK cell cytotoxicity mainly through IL-12-mediated IFN-γ production using gene-targeted mice and neutralizing mAbs.

A. blazei has been taken orally as one of the most common CAMs for cancer and other diseases.[1–3] To examine the effector mechanisms possibly induced by *A. blazei* in humans, we used a mouse experimental model employing oral administration of *A. blaze* or either extract. The results obtained *in vivo* experiments were consistent with those obtained *in vitro*. Thus, the presented results strongly suggest that the *in vivo* NK cell activation mechanisms of *A. blazei* in human would likely be quite similar to that revealed *in vitro* experiments.

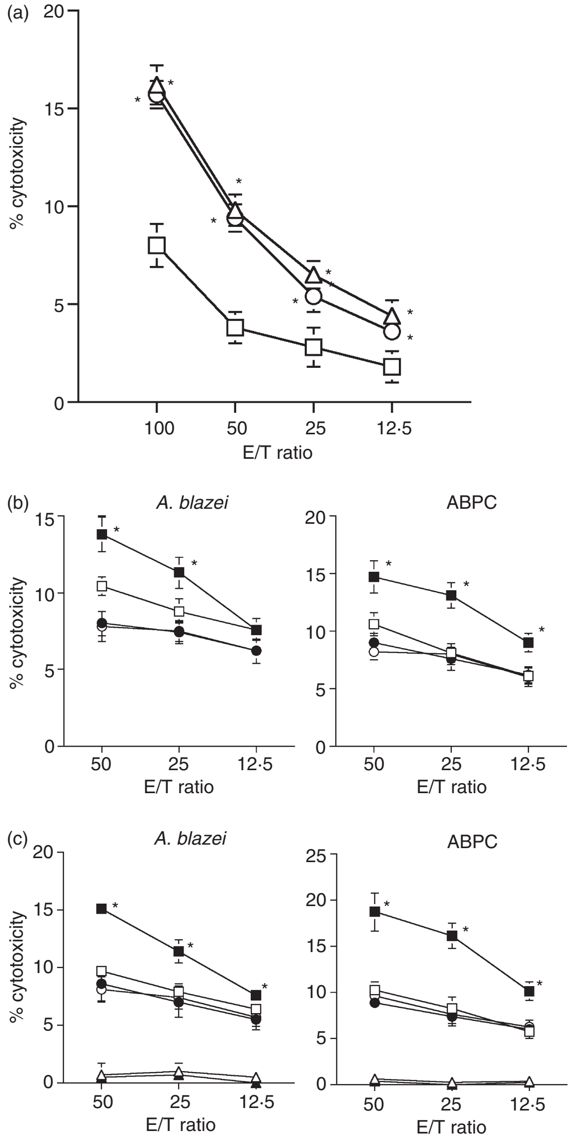

Figure 7. IL-12 mediated IFN-γ-dependent NK cell cytotoxicity induced by an extract of *A. blazei* or ABPC *in vitro*. (a) Augmentation of NK cell cytotoxicity by an extract of *A. blazei* or ABPC. DC-rich spleen MNC isolated from B6 mice were cultured with an extract of *A.blazei* (0·1%) (open triangle), ABPC (0·05%) (open circle), or control water (open square) for 72 hr. Cells were then harvested and cytotoxic activity was examined using the NK cell-sensitive target, YAC-1, at the indicated ratios. $^{*}P < 0{\cdot}05$. (b) IFN-γ dependent augmentation of cytotoxicty by an extract of *A. blazei* or ABPC. DC-rich spleen MNC from WT (square) or IFN-$\gamma^{-/-}$ (circle) B6 mice were cultured in the presence (closed) or absence (open) of an extract of *A.blazei* (0·1%) or ABPC (0·05%) for 72 hr. Cells were then harvested and cytotoxic activity was examined. $^{*}P < 0{\cdot}05$ when the cytotoxic activity of stimulated MNC is compared with that of control MNCs. (c) IL-12-dependent augmentation of NK cell cytotoxicty by an extract of *A. blazei* or ABPC. DC-rich spleen MNCs isolated from B6 mice were cultured in the presence (closed) or absence (open) of an extract of *A.blazei* (0·1%) or ABPC (0·05%) with anti-IL-12 mAb (circle) or control immunoglobulin (square). After 72 hr incubation, cells were harvested and cytotoxic activity was analysed. In some experiments, NK cells were depleted from DC-rich MNC stimulated without anti-IL-12 mAb before cytotoxic assay (triangle). $^{*}P < 0{\cdot}05$ when the cytotoxic activity is compared with that of control MNC. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in two or three independent experiments.

NK cells and IFN-γ are recognized as critical for immune surveillance for tumour and pathogen.[12–15,26,27] IL-12 and IL-18 have been reported to activate NK cells through the induction of IFN-γ production[28–30] and IFN-γ and IL-12 induction by the extract of *A. blazei* has been reported previously.[10,17,20] In the present study, we demonstrated oral administration of *A. blazei* augmented NK cell cytotoxicity, which is commonly believed to be the major effector mechanism of NK cells against tumours and microbes.[14,15,31,32] In contrast, it was also reported that IFN-γ produced by IL-12-activated NK and/or NKT cells induced NO-mediated cytotoxicity and/or antiangiogenic chemokines, and these pathways played a substantial role in the antitumour effect of IL-12.[28,33–35] IFN-γ has been suggested to play a substantial role in the immunomodulatory effect of *A. blazei*.[5,10,17] Direct induction of tumour apoptosis, inhibition of tumour cell proliferation, and inhibition of tumour-induced neovascularization have been reported as the possible mechanisms of the antitumour effect of *A. blazei*.[1,8,11,16] Nevertheless the contribution of IFN-γ to these effects has not been examined. It was also reported that *A. blazei* induced several cytokine and chemokine genes (including IL-1 and IL-8) in a human monocyte cell line (THP-1).[36] Thus, several mechanisms could contribute to the antitumour effect of *A. blazei* as well as NK cell-mediated direct cytotoxicity against tumour cells.

Here we demonstrated that IL-12-mediated IFN-γ induction by *A. blazei* or ABPC is TLR-4 independent. It was suggested that β-glucan might be the effector component that induces immune responses by *A. blazei* and ABPC,[5–7,37] although we do not have any evidence showing β-glucan as the effector component inducing IL-12 and IFN-γ-mediated NK cell activation and we have not yet identified the structure of β-glucans contained in *A.blazei* or ABPC. Dectin-1 was recently reported as the receptor for β(1→3)- and/or β(1→6)-linked glucan- and zymosan-inducing IL-12 production.[38] Complement receptor 3, lactosylceramide, and scavenger receptors are also reported as β-glucan receptors inducing immune responses.[39] TLR-2- and TLR-6-mediated signals are reported to be required for optimal β-glucan-induced and dectin-1-mediated immune responses;[40,41] however, ligands of TLR-2 or TLR-6 are not still identified in β-glucans or zymogen. Thus, dectin-1, TLR-2, and TLR-6 would co-operate in recognition of β-glucans and activate immune responses.[39] Complex signalling mechanisms

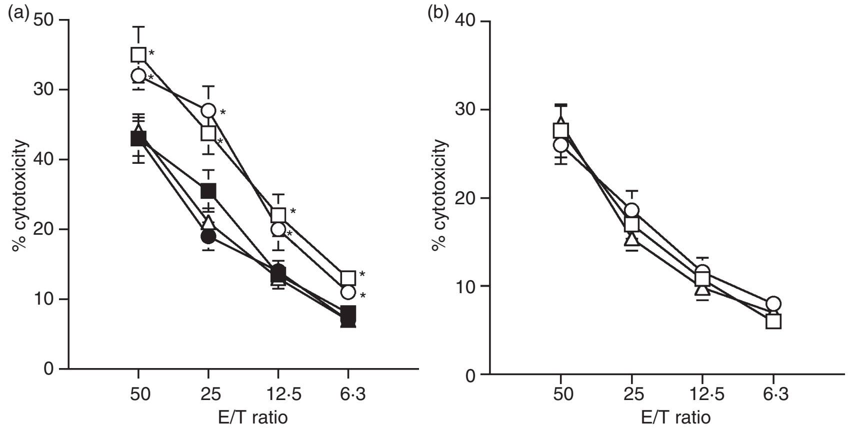

Figure 8. IL-12-mediated activation of NK cell cytotoxicity induced by an extract of *A. blazei* or ABPC *in vivo*. (a) WT B6 mice were administered daily with *A. blazei* extract (500 μl/head) (square), ABPC extract (250 μl/head)(circle) or water (500 μl/head) (triangle) for 2 weeks. Some mice were also administrated with 300 μg of anti-IL-12 mAb (closed) or control immunoglobulin (open) every 3 days. Then, liver MNCs were prepared and cytotoxic activity was analysed using NK cell-sensitive target cells, YAC-1, at the indicated E/T ratios. *$P < 0{\cdot}05$ when the cytotoxic activity is compared with that of control MNCs from water-administered mice. (b) IFN-$\gamma^{-/-}$ B6 mice were administered daily with *A. blazei* extract (500 μl/head) (square), ABPC extract (250 μl/head) (circle) or water (500 μl/head) (triangle) for 2 weeks. Then, liver MNCs were prepared and cytotoxic activity was analysed using NK cell-sensitive target cells, YAC-1, at the indicated E/T ratios. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in two or three independent experiments.

would underlie immune activation by β-glucans; however, further investigations to identify the receptors and signalling mechanisms involved in the recognition of β-glucans are required. This will not only elucidate immune activation mechanisms against fungal pathogens but also improve the therapeutic benefit of mushroom-derived CAMs.

We now have reported that IL-18 partly contributes to *A. blazei*-induced, but not ABPC-induced, IFN-γ production, and ABPC demonstrated a two times higher efficacy in IL-12-mediated IFN-γ induction compared with *A. blazei*. These results suggested that ABPC preparations may have retained effective components inducing IL-12 production, but lost those inducing IL-18 production. However, the enhancement of NK cell cytotoxicity and the amount of IFN-γ induced by *A. blazei* or ABPC was significantly less than that triggered by recombinant IL-12 *in vitro* and *in vivo*, suggesting limited production of IFN-γ-inducing cytokines (IL-12 and/or IL-18) after *A. blazei* or ABPC treatment. We reported that IFN-γ-induced perforin-dependent NK cell cytotoxicity is the critical for the antimetastatic effect of recombinant IL-12.[42] Thus, the NK cell cytotoxicity-dependent antimetastatic effect might be less after *A. blazei* or ABPC treatment compared with that induced by exogenous recombinant IL-12 treatment. However, it is possible that the amount of IL-12 and IFN-γ induced by *A. blazei* or ABPC might be sufficient to play a role in the surveillance for tumour development. Alternatively, IFN-γ independent antitumour mechanisms might play a role together with the IFN-γ-mediated antitumour effects of *A. blazei*. IL-12, IL-18, and IFN-γ were reported to induce hepatotoxicity and act as critical cytokines in the Shwartzman reaction[43,44] suggesting that continuous production of large amounts of these cytokines might be toxic. Moreover, the tumour promotion effect of some other commercial products of *A. blazei* was recently reported by the Food Safety Commission of Japan, although the products used here were proved as safe (data not shown). Thus, further precise studies to identify the effective components of *A. blazei* and ABPC that induce IL-12 and/or IL-18, their downstream mechanisms activating immune responses against tumours, and toxicity are required to improve their therapeutic effects.

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